Immunity
Article

E2A Proteins Promote Development of Lymphoid-Primed Multipotent Progenitors

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DOI 10.1016/j.immuni.2008.05.015

SUMMARY

The first lymphoid-restricted progeny of hematopoietic stem cells (HSCs) are lymphoid-primed multipotent progenitors (LMPPs), which have little erythromyeloid potential but retain lymphoid, granulocyte, and macrophage differentiation capacity. Despite recent advances in the identification of LMPPs, the transcription factors essential for their generation remain to be identified. Here, we demonstrated that the E2A transcription factors were required for proper development of LMPPs. Within HSCs and LMPPs, E2A proteins primed expression of a subset of lymphoid-associated genes and prevented expression of genes that are not normally prevalent in these cells, including HSC-associated and nonlymphoid genes. E2A proteins also restricted proliferation of HSCs, MPPs, and LMPPs and antagonized differentiation of LMPPs toward the myeloid fate. Our results reveal that E2A proteins play a critical role in supporting lymphoid specification from HSCs and that the reduced generation of LMPPs underlies the severe lymphocyte deficiencies observed in E2A-deficient mice.

INTRODUCTION

Lymphocyte development from hematopoietic stem cells (HSCs) is accompanied by a loss of self-renewal capacity and a progressive restriction of developmental potential. The pathway to the lymphocyte fate involves the generation of multipotent progenitors (MPPs) and lymphoid-primed multipotent progenitors (LMPPs) that have lymphoid as well as granulocyte and macrophage progenitor (GMP) potential but little or no megakaryocyte and erythrocyte progenitor (MEP) potential, respectively (Adolfsson et al., 2005; Lai et al., 2005). However, the precise stage in which MEP potential is lost remains controversial (Forsberg et al., 2006). LMPPs are precursors to common lymphoid progenitors (CLPs), which have lost GMP potential but retain multilineage lymphoid-differentiation capacity when placed in an appropriate microenvironment (Kondo et al., 1997). LMPPs also differentiate into early T lymphocyte progenitors (ETPs), which undergo T lymphocyte lineage specification upon activation of Notch receptors by Notch ligands in the thymus (Allman et al., 2003; Sambandam et al., 2005). Although the molecular mechanisms for generation of committed B or T lymphocytes are beginning to be revealed, a major unanswered question is how the multipotent progeny of HSCs initiate differentiation toward lymphoid fate.

HSCs, MPPs, and LMPPs are lineage$^-$Sca-1$^+$c-*kit*^{hi} (LSK) cells that can be distinguished by the increasing expression of the transmembrane receptor *fms*-like tyrosine kinase 3 (Flt3) (Adolfsson et al., 2001; Adolfsson et al., 2005). Upregulation of Flt3 is associated with decreased megakaryocyte- (Mk) and erythrocyte (E)-associated gene expression. Lymphoid-associated gene expression is initiated in LMPPs, indicating priming of the lymphoid gene program (Igarashi et al., 2002; Mansson et al., 2007). A small number of transcriptional regulators have been implicated in the development or function of LMPPs. The zinc-finger transcription factors encoded by the *Ikzf1* (Ikaros) gene are dispensable for the generation of LMPPs but required for high expression of Flt3 and subsequent lymphocyte differentiation (Yoshida et al., 2006). The Ets-related transcription factor PU.1, encoded by *Sfpi1*, is increased in a subset of LMPPs, and Flt3^{hi} LSKs are absent in the liver of *Sfpi1*$^{-/-}$ embryos and the bone marrow (BM) of adult mice with a conditional deletion of *Sfpi1* (Arinobu et al., 2007; Dakic et al., 2005; Iwasaki et al., 2005). However, it remains to be determined whether PU.1 is required for development of functional LMPPs or, like Ikaros, is required specifically for Flt3 expression (Dakic et al., 2005; Iwasaki et al., 2005). Aside from these two factors, little is known about the transcriptional regulators that specify differentiation toward LMPPs in multipotent progenitors (Nutt and Kee, 2007).

Lymphoid priming involves the induction of lymphoid genes, including *Rag1*, *Dntt*, and *Igh-6*, which are known targets of the basic helix-loop-helix (bHLH) transcription factors E12 and E47, encoded by *Tcfe2a* (E2A) (Kee and Murre, 1998, 2001; Mansson et al., 2007). The E-box-binding transcription factors (E proteins) function in cell-fate specification and differentiation in diverse cell types in both invertebrates and vertebrates (Massari and Murre, 2000). The E2A proteins have been characterized extensively as regulators of B and T lymphocyte development and are thought to function in specification of these lineages from lymphoid-restricted multipotent progenitors (Nutt and Kee, 2007; Welner et al., 2008). E2A proteins specify the B lymphocyte fate by regulating the essential transcription factor encoded by the early B cell factor 1 (*Ebf1*) gene, in collaboration

with PU.1 and interleukin (IL)-7 receptor-activated STAT5 (Dias et al., 2005; Kee and Murre, 1998; Kikuchi et al., 2005; Medina et al., 2004; Roessler et al., 2007; Seet et al., 2004). Although no B lymphocytes are generated in *Tcfe2a*$^{-/-}$ mice, T lymphocytes develop, albeit in reduced numbers (Bain et al., 1997a; Bain et al., 1994; Zhuang et al., 1994). In vitro studies suggest that E2A proteins regulate expression of the essential T lymphocyte specification factor *Notch1* and collaborate with Notch1 to activate target genes such as *Hes1* (Ikawa et al., 2006). However, the requirements for E2A in vivo and in vitro differ because E2A is indispensable for T lymphopoiesis in vitro on OP9 stromal cells expressing the Notch ligand Delta-like 1, but T cells develop in vivo with a partial arrest at the DN1-to-DN2 transition (i.e., after the initial requirement for Notch1) (Bain et al., 1997a; Ikawa et al., 2006; Kee et al., 2002). Therefore, the role of E2A proteins in early T lymphocyte development in vivo remains to be fully understood.

Multipotent hematopoietic progenitor cell lines can be generated from *Tcfe2a*$^{-/-}$ BM, suggesting a role for E2A proteins in preventing expansion of multipotent cells and promoting differentiation (Ikawa et al., 2004). However, aside from their known roles in the regulation of B and T lymphocyte genes, a function for E2A proteins in earlier stages of hematopoiesis has not been reported. We show here that E2A proteins promote development of LMPPs and are required to establish an appropriate LMPP transcriptome. Importantly, E2A appears to be dispensable for extinction of the MEP fate en route to LMPPs but restricts proliferation and further myeloid differentiation in LMPPs. Our results reveal a dose-dependent requirement for E2A proteins at the earliest stages of lymphoid specification and indicate that reduced thymopoiesis in *Tcfe2a*$^{-/-}$ mice initiates from a failure to produce sufficient numbers of functional BM-derived LMPPs.

RESULTS

A Dose-Dependent Requirement for E2A in Development of CLPs and ETPs

E2A proteins are required for B cell development in part because they promote expression of EBF1 (Kee and Murre, 1998; Seet et al., 2004). This observation contributed to the prevailing view that E2A proteins play a critical role at the CLP-to-pro-B-lymphocyte transition, during which EBF1 first becomes essential (Pongubala et al., 2008; Welner et al., 2008). A similar view has developed regarding the requirements for E2A in T cell development, during which these proteins are thought to be essential for commitment to the T lymphocyte lineage, despite recent evidence implicating E2A in the regulation of *Notch1*, which is required already by the ETP stage (Bain et al., 1997a; Ikawa et al., 2006; Kee et al., 2002; Rothenberg, 2007). However, it has not been determined whether E2A proteins function at earlier stages of lymphohematopoiesis. Therefore, we undertook a rigorous quantitative analysis of lymphocyte-progenitor populations in *Tcfe2a*$^{+/+}$, *Tcfe2a*$^{+/-}$, and *Tcfe2a*$^{-/-}$ mice by flow cytometry. Consistent with a previous report (Borghesi et al., 2005), we found a significant reduction in the percentage and total number of CLPs in *Tcfe2a*$^{-/-}$ as compared to *Tcfe2a*$^{+/+}$ BM (90%, $p < 0.001$). Interestingly, however, we also found a reduced number of *Tcfe2a*$^{+/-}$ CLPs (60%, $p < 0.001$), indicating a dose-dependent requirement for E2A at this stage of lymphopoiesis (Figures 1A and 1B). Analysis of Lin$^-$ thymocytes revealed a dose-dependent requirement for E2A in development of ETPs and DN2 thymocytes (Figures 1C and 1D). However, the number of *Tcfe2a*$^{+/-}$ DN3 thymocytes is indistinguishable from that of the wild-type (WT), whereas *Tcfe2a*$^{-/-}$ DN3 cells remain reduced, although the number was variable (Figures 1C and 1D). The reduced number of ETPs in *Tcfe2a*$^{+/-}$ and *Tcfe2a*$^{-/-}$ mice was not revealed in previous studies because of contamination of the conventional DN1 subset with non-T lineage cells, which are not affected by deletion of E2A (Allman et al., 2003; Bain et al., 1997a; Kee et al., 2002). Therefore, we conclude that there is a dose-dependent requirement for *Tcfe2a* during development of CLPs, ETPs, and DN2 thymocytes.

A Dose-Dependent Requirement for E2A in Development of LMPPs

E2A proteins are widely expressed in BM multipotent hematopoietic progenitors (Figure S1 available online) (Zhuang et al., 2004), and our data indicate that E2A may be required upstream of CLPs and ETPs. Therefore, we undertook a quantitative analysis of BM LSK cells, including HSCs, MPPs, and LMPPs in *Tcfe2a*$^{+/+}$, *Tcfe2a*$^{+/-}$, and *Tcfe2a*$^{-/-}$ mice. We found that the frequency and total number of LSK cells was decreased in *Tcfe2a*$^{+/-}$ and *Tcfe2a*$^{-/-}$ compared to *Tcfe2a*$^{+/+}$ BM (Figures 2A and 2B). The number of HSCs and MPPs was not significantly different between each of these strains, although a consistent mild decrease was observed (Figures 2A and 2B). In contrast, the number of LMPPs was significantly reduced in both *Tcfe2a*$^{+/-}$ (68%, $p = 0.008$) and *Tcfe2a*$^{-/-}$ (84%, $p = 0.002$) BM compared to *Tcfe2a*$^{+/+}$ BM (Figures 2A, 2B, and 2C). The decreased frequency of LMPPs did not appear to be the consequence of a requirement for E2A in transcription of the *Flt3* gene because there is no compensatory increase in the number of Flt3$^-$ or Flt3^{lo} LSKs (Figure 2B). In addition, we have found no evidence that E2A can induce *Flt3* mRNA in MPPs under conditions in which E2A can induce other potential target genes (see below). We also found a reduced frequency of VCAM-1$^-$ LSKs in *Tcfe2a*$^{+/-}$ and *Tcfe2a*$^{-/-}$ BM (Figure 2A), further supporting the conclusion that LMPPs are reduced in *Tcfe2a*$^{+/-}$ and *Tcfe2a*$^{-/-}$ mice (Lai and Kondo, 2006; Lai et al., 2005). In contrast, erythromyeloid progenitors were not decreased, but rather, they were slightly increased in *Tcfe2a*$^{+/-}$ and *Tcfe2a*$^{-/-}$ mice (Figure S2). Taken together, our data indicate that E2A proteins play a dose-dependent role in the development of LMPPs, which represent the earliest lymphoid-primed progeny of HSCs.

E2A Proteins Antagonize Proliferation of HSCs, MPPs, and LMPPs

E proteins are necessary for pro-B lymphocyte proliferation (Kee, 2005; Seet et al., 2004), although high concentrations of E2A can restrain proliferation (Engel and Murre, 2004). Therefore, we considered the possibility that LMPPs are reduced in number in *Tcfe2a*$^{+/-}$ and *Tcfe2a*$^{-/-}$ mice because these cells or their precursors require E2A for expansion. To test this possibility, we examined BrdU incorporation in *Tcfe2a*$^{+/+}$ and *Tcfe2a*$^{-/-}$ BM LSK subpopulations after 24 hr of in vivo labeling. Surprisingly, we found that *Tcfe2a*$^{-/-}$ LSKs incorporated more BrdU than *Tcfe2a*$^{+/+}$ LSKs (Figure 3A). Importantly, all *Tcfe2a*$^{-/-}$ LSK

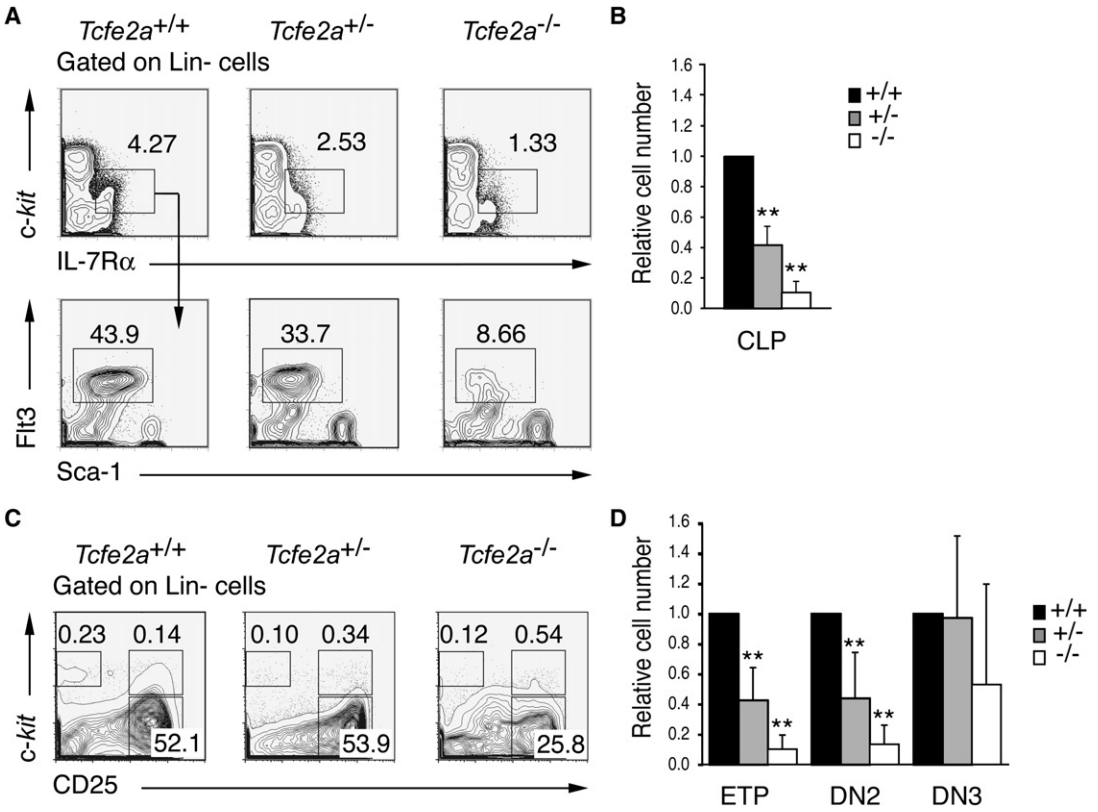

Figure 1. A Dose-Dependent Requirement for E2A in the Generation of CLPs, ETPs, and DN2 Cells

(A) Lin$^-$ BM cells from *Tcfe2a*$^{+/+}$, *Tcfe2a*$^{+/-}$, and *Tcfe2a*$^{-/-}$ mice were analyzed for surface expression of c-*kit* and IL-7Rα (upper panels), and the *c-kit*^{lo}IL-7Rα$^+$ subpopulation was analyzed for expression of Sca-1 and Flt3 (lower panels) by flow cytometry. CLPs are Lin$^-$c-*kit*^{lo}IL-7Rα$^+$Sca-1$^+$Flt3$^+$.

(B) Relative number of CLPs in BM of adult *Tcfe2a*$^{+/-}$ (gray) and *Tcfe2a*$^{-/-}$ (white) mice as compared to wild-type mice (black; set to 1). A minimum of five mice were analyzed in each group. Error bars represent the mean ± SD; **p < 0.01.

(C) Surface expression of c-*kit* and CD25 on Lin$^-$ thymocytes; numbers represent the percentage of cells in each gate.

(D) Relative number of ETPs, DN2, and DN3 cells in the thymus of adult *Tcfe2a*$^{+/-}$ (gray) and *Tcfe2a*$^{-/-}$ (white) mice as compared to wild-type mice (black; set to 1). A minimum of five mice were analyzed in each group. Error bars represent the mean ± SD; ** p < 0.01.

subsets incorporated more BrdU than the corresponding *Tcfe2a*$^{+/+}$ populations (Figure 3B). Therefore, the reduced number of LMPPs in *Tcfe2a*$^{+/-}$ and *Tcfe2a*$^{-/-}$ BM was not a consequence of reduced proliferation of LMPPs or their precursors. Indeed, E2A proteins appeared to limit proliferation of these multipotent cells. Importantly, after in vitro culture, *Tcfe2a*$^{-/-}$ and *Tcfe2a*$^{+/+}$ LSKs showed a similar or slightly reduced frequency of apoptotic cells as measured by annexin V binding, indicating that the lack of E2A is not leading to increased cell death (Figure 3C). These data lead us to suggest that *Tcfe2a*$^{-/-}$ LSK cells preferentially adopt a fate other than LMPP.

E2A Proteins Are Required for Proper Lymphoid Gene Expression in LMPPs

To further address the requirements for E2A in the development of multipotent lymphoid progenitors, we examined the transcriptome of purified *Tcfe2a*$^{+/+}$ and *Tcfe2a*$^{-/-}$ LMPPs. Differentially expressed genes were further characterized on the basis of their expression in *Tcfe2a*$^{+/+}$ HSCs, as well as in long-term (LT) HSC, preGM, CLPs, megakaryocyte progenitors (MkP), and pre-colony-forming unit (CFU)-E (Pronk et al., 2007). Comparing overall gene expression in *Tcfe2a*$^{+/+}$ and *Tcfe2a*$^{-/-}$ LMPPs, the Pearson correlation was 0.99 as compared to 0.94 when either LMPP sample was compared to *Tcfe2a*$^{+/+}$ HSCs. Therefore, the few Flt3^{hi} LSKs in *Tcfe2a*$^{-/-}$ mice are LMPPs, and any alterations in gene expression are unlikely to result from differences in cell composition. A total of 47 genes failed to be appropriately expressed (i.e., decreased by >50%) in *Tcfe2a*$^{-/-}$ LMPPs, indicating that these genes are directly or indirectly regulated by E2A (Figure 4A). Twenty-six of these genes were more highly expressed in CLPs than in other progenitor populations, indicating that E2A proteins play a major role in establishing the lymphoid-associated gene-expression signature. Among these genes were multiple known E2A targets, including *Rag1*, *Il7r*, *Dntt, Igh-6*, and *Notch1* (Figure 4A). Some of these lymphoid-associated genes were found to be expressed in HSCs and dependent on E2A, indicating that they are already being primed in the HSC population in an E2A-dependent manner (Table 1). These genes are candidates for genes that influence lymphoid specification (Georgopoulos, 2002).

In addition to the set of known E2A target genes, multiple genes were decreased in *Tcfe2a*$^{-/-}$ LMPPs that have not been previously shown to be E2A dependent (Figure 4A and Figure S3). One of these genes is *Ccr9*, which encodes the

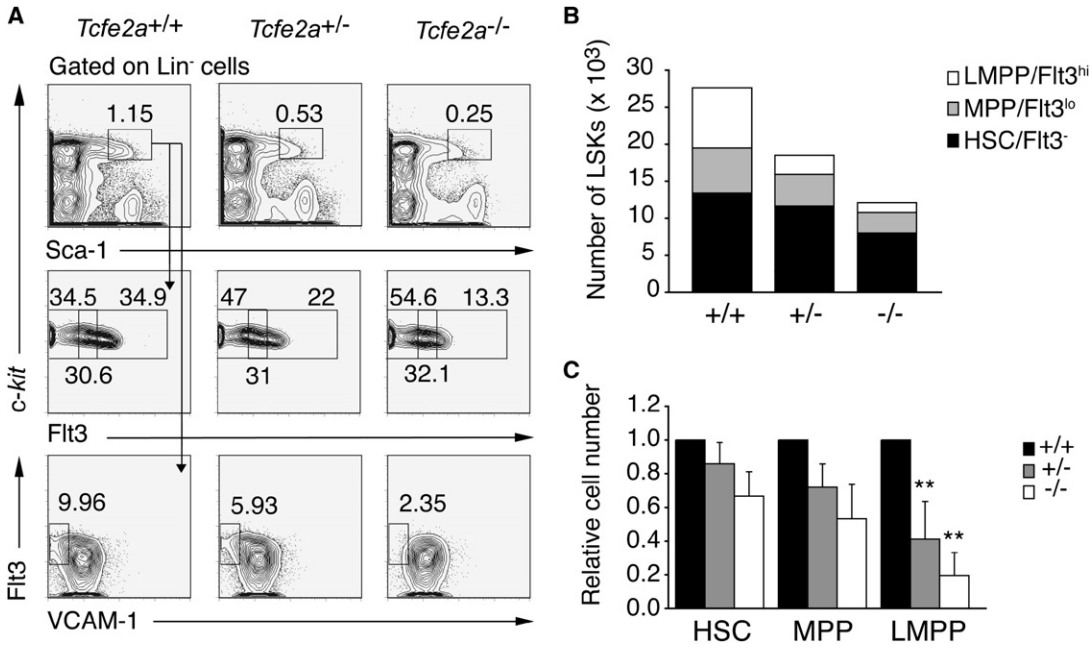

Figure 2. A Dose-Dependent Requirement for E2A in the Generation of LMPPs

(A) Lin$^-$ BM cells from *Tcfe2a*$^{+/+}$, *Tcfe2a*$^{+/-}$, and *Tcfe2a*$^{-/-}$ mice were analyzed for surface expression of c-*kit* and Sca-1 (upper panels). The LSK subset was analyzed for expression of c-*kit* and Flt3 (middle panels) or Flt3 and VCAM-1 (lower panels). Numbers represent the percent of cells in the indicated gate. LMPPs are LSK Flt3^{hi} and LSK VCAM-1$^-$.

(B) The total number of HSCs (black), MPPs (gray), and LMPPs (white) in *Tcfe2a*$^{+/+}$, *Tcfe2a*$^{+/-}$, and *Tcfe2a*$^{-/-}$ mice is shown, and the sum of these populations is the total number of LSK cells.

(C) Number of HSCs, MPPs, LMPPs, and CLPs in *Tcfe2a*$^{+/-}$ (gray) and *Tcfe2a*$^{-/-}$ (white) mice relative to the WT (black; set to 1). A minimum of six mice were analyzed in each group. Error bars represent the mean ± SD; **p < 0.01.

receptor for the chemokine CCL25, and we confirmed that *Ccr9* and *Notch1* mRNA were reduced in *E2A*$^{+/-}$ and *E2A*$^{-/-}$ LMPPs by quantitative PCR (Figure 4B). Importantly, *Ccr9,* but not *Flt3* mRNA, could be induced by E47 in *Tcfe2a*$^{-/-}$ progenitors cultured in vitro (Figure 4C). CCR9 is expressed on a small population of LMPPs that possess efficient in vivo thymus-repopulating capacity (Benz and Bleul, 2005; Schwarz et al., 2007; Scimone et al., 2006), and this subpopulation is severely decreased in both *Tcfe2a*$^{+/-}$ and *Tcfe2a*$^{-/-}$ mice (92%, p = 0.021 and 96%, p = 0.014, respectively) (Figures 4D and 4E). Therefore, the loss of *Ccr9*-expressing LMPPs is likely to contribute to the reduced number of ETPs observed in *Tcfe2a*$^{+/-}$ and *Tcfe2a*$^{-/-}$ mice.

E2A Does Not Regulate Expression of Key Lymphoid Transcription Factors in LMPPs

The microarray analysis of *Tcfe2a*$^{+/+}$ and *Tcfe2a*$^{-/-}$ LMPPs revealed a central role for E2A in lymphoid priming. Importantly, however, E2A proteins were not required for expression of the key lymphoid transcription factors Ikaros, PU.1, and Gfi1, which are essential for lymphocyte development beyond the LMPP stage (Figure 5A) (Hock et al., 2004; Medina et al., 2004; Yoshida et al., 2006; Zeng et al., 2004). Nonetheless, known targets of PU.1 and Ikaros, including *Dntt* and *Il7r*, were not expressed appropriately (Figure 4A). An analysis of the genomic DNA surrounding the transcription start sites of CLP-associated E2A-dependent genes revealed that all but two of these genes (90%) have conserved (between human and mouse) potential binding sites for E2A (Figure 5B). Moreover, more than 60% of these genes had potential binding sites for E2A, Ikaros, and/or PU.1 (Figure 5B). In contrast, within a set of randomly selected genes that were expressed equivalently in *Tcfe2a*$^{+/+}$ and *Tcfe2a*$^{-/-}$ LMPPs, 40% lacked potential E2A binding sites and only 25% had sites for E2A, Ikaros, and/or PU.1. Taken together, these data indicate that E2A is required for Ikaros and PU.1 to promote expression of at least a subset of their target genes in LMPPs.

E2A Proteins Restrict the GMP Potential, but Not the MEP Potential, of LMPPs

Although E2A is required for the expression of multiple LMPP-associated genes, we found that there is a set of genes that is expressed more abundantly in *Tcfe2a*$^{-/-}$ compared to *Tcfe2a*$^{+/+}$ LMPPs, including genes expressed in HSCs, preGM, MkP, and pre-CFU-E (Figure 4A and Figure S3). A subset of these genes is also expressed more abundantly in *Tcfe2a*$^{-/-}$ HSCs, indicating that these alterations in gene expression initiate within the HSC compartment and may influence lymphoid specification (Table 1). These data also show that the few *Tcfe2a*$^{-/-}$ LMPPs that develop have a substantially altered transcriptome and fail to appropriately extinguish a set of non-LMPP-associated genes.

Our data indicate that in the absence of E2A, there is a progressive loss of lymphoid progenitor potential that initiates within the HSC or MPP compartment and that becomes apparent by the time of the emergence of LMPPs. The transition from HSC to LMPP is associated with a progressive loss of MEP

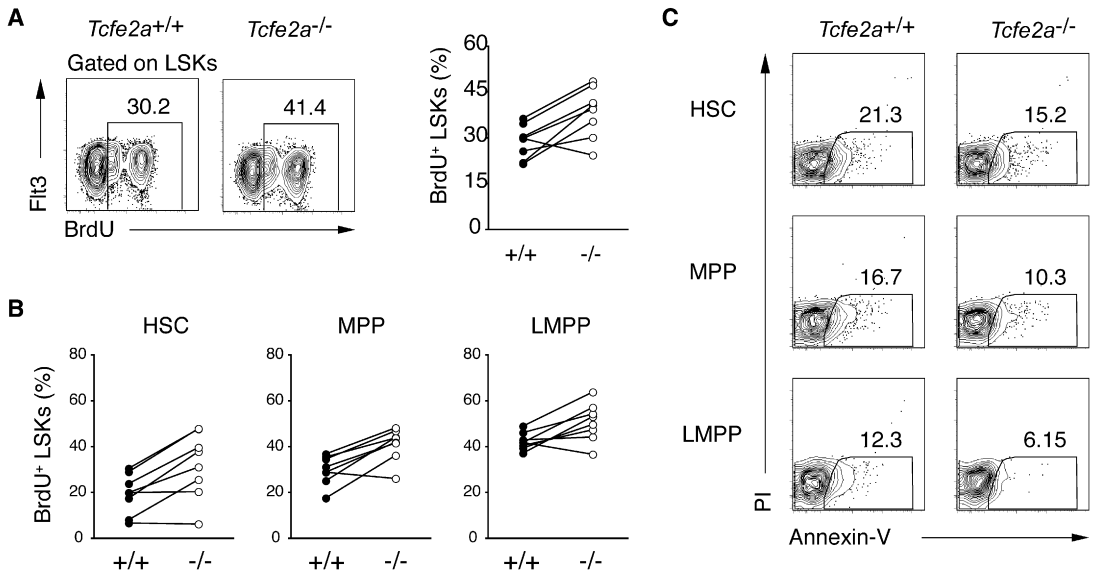

Figure 3. E2A Proteins Restrict HSC, MPP, and LMPP Proliferation

(A) BrdU incorporation in BM LSKs as determined after 24 hr of in vivo exposure to BrdU. LSKs were analyzed for surface expression of Flt3 and intracellular BrdU; numbers indicate the percentage of $BrdU^+$ LSKs. The graph summarizes results of five independent experiments.

(B) Percentage of HSCs, MPPs, and LMPPs that were $BrdU^+$ in *Tcfe2a*$^{+/+}$ and *Tcfe2a*$^{-/-}$ mice, respectively.

(C) Representative FACS analysis for annexin-V and PI staining on cultured HSC, MPP, or LMPPs cells. Numbers represent the percentage of cells in each plot that are positive for annexin V, as shown in the gated region.

developmental potential (Adolfsson et al., 2005; Mansson et al., 2007). Therefore, we questioned whether the loss of E2A influences the erythromyeloid potential of MPPs or LMPPs. To test this possibility, we examined the ability of single *Tcfe2a*$^{+/+}$ and *Tcfe2a*$^{-/-}$ HSCs, MPPs, and LMPPs to give rise to Mk in vitro under conditions that efficiently support Mk and other myeloid lineage development (Figure 6A). As reported previously (Adolfsson et al., 2005; Arinobu et al., 2007), we found that WT HSCs, MPPs, and LMPPs have a high cloning efficiency but that LMPPs do not develop into Mk under these conditions (Figures 6B and 6C). Importantly, sorted *Tcfe2a*$^{-/-}$ HSCs, MPPs, and LMPPs showed a similar restriction of Mk potential (Figure 6B). However, *Tcfe2a*$^{-/-}$ LMPPs had a higher clongenic potential than their WT counterparts, and individual *Tcfe2a*$^{-/-}$ clones were markedly larger than WTs (Figure 6D). *Tcfe2a*$^{-/-}$ MPP colonies were also, on average, larger than *Tcfe2a*$^{+/+}$ MPP-derived colonies (Figure 6D). Using a methycellulose-based assay, we also observed that $Flt3^+$ LSKs from *Tcfe2a*$^{-/-}$ mice have an increased clonogenic potential compared to *Tcfe2a*$^{+/+}$ cells (Figure 6D). Wright-Giemsa staining of individual clones under both culture conditions revealed that WT and *Tcfe2a*$^{-/-}$ LMPPs gave rise only to granulocytes and/or macrophages (Figure 6C and Figure S4). We note that in *Tcfe2a*$^{-/-}$ LMPP clones, we also detected an increase in atypical granulocytes, which appear to be cells undergoing apoptosis, suggesting that loss of E2A may influence the rate of granulocyte differentiation or the lifespan of granulocytes under these conditions (Figure S4). Taken together, our data support a model in which E2A proteins promote differentiation toward the lymphoid fate and, in the absence of E2A, MPPs and LMPPs preferentially adopt the GMP fate. This model is consistent with previous studies showing that E2A proteins can induce lymphoid-lineage conversion in a macrophage cell line and that E2A represses macrophage development from MPPs in vitro (Bhalla et al., 2008; Kee and Murre, 1998).

DISCUSSION

We have identified a requirement for E2A proteins in supporting the development of LMPPs from HSCs. E2A proteins are essential for the initial priming of a subset of lymphoid-associated genes, some of which are already detected within HSCs. In the absence of E2A, key lymphoid-promoting transcription factors, such as PU.1, Ikaros, and Gfi1, are expressed appropriately; however, E2A is necessary for these genes to activate some of their targets in LMPPs. Moreover, we found that E2A-dependent lymphoid genes frequently have conserved potential binding sites for PU.1 and/or Ikaros, indicating that these transcription factors may cooperatively regulate the lymphoid gene program. Nonetheless, the role of E2A extends beyond the induction of lymphoid genes because *Tcfe2a*$^{-/-}$ HSCs and LMPPs showed aberrant expression of genes that are not normally expressed in these cells. We also found that E2A proteins restrict proliferation of HSCs, MPPs, and LMPPs and antagonize GMP differentiation from LMPPs in vitro. Our data indicate that the first essential function of E2A proteins in lymphocyte development is to support the development of a lymphoid-primed cell population that is capable of further restriction to specific lymphoid fates.

Our data reveal a severe loss of LMPPs in both *Tcfe2a*$^{+/-}$ and *Tcfe2a*$^{-/-}$ mice. However, unlike Ikaros and PU.1, which are also required for the development of $Flt3^{hi}$ LSKs, we found no evidence that E2A proteins directly regulate *Flt3* (Medina et al., 2004; Yoshida et al., 2006). *Flt3* mRNA was not differentially

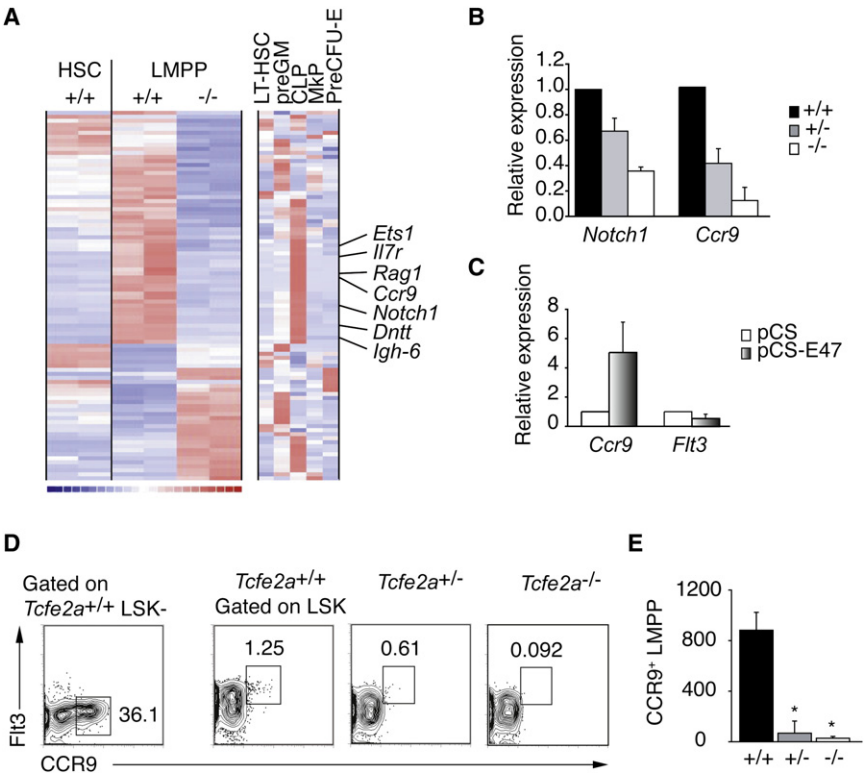

Figure 4. Analysis of the *Tcfe2a*$^{+/+}$ and *Tcfe2a*$^{-/-}$ LMPP Transcriptome

(A) Clustering of genes that are differentially expressed in replicate samples (one per column) of *Tcfe2a*$^{+/+}$ and *Tcfe2a*$^{-/-}$ LMPPs (Affymetrix MOE430 2.0 arrays). Expression of these genes in *Tcfe2a*$^{+/+}$ HSCs is shown for comparison. The clustering includes all genes with expression levels >50 in at least one of the four LMPP arrays and differing by a factor of two (with a lower 90% confidence bound of fold change). Each row corresponds to one unique identifier, and a subset of CLP-associated genes is indicated (see Figure S3 for complete list of genes). The rightmost clustering shows the lineage association of these differentially expressed genes in LT-HSC, pre-GM, CLP, MkP, and pre-CFU-E (as defined in Pronk et al. [2007]). Red indicates high expression levels, blue indicates low expression levels, and white indicates intermediate expression levels.

(B) Relative expression of *Notch1* and *Ccr9* mRNA in purified *Tcfe2a*$^{+/-}$ and *Tcfe2a*$^{-/-}$ LMPPs, as compared to *Tcfe2a*$^{+/+}$ progenitors (normalized to *Hprt* and set to 1), determined by quantitative polymerase chain reaction (QPCR). Error bars represent the mean ± SD of replicate experiments.

(C) QPCR analysis of *Ccr9* and *Flt3* mRNA in *Tcfe2a*$^{-/-}$ fetal-liver multipotent progenitors, 36 hr after transduction with control or E47-producing retrovirus. One of two experiments is shown. Error bars represent the mean ± SD.

(D) *Tcfe2a*$^{+/+}$, *Tcfe2a*$^{+/-}$, and *Tcfe2a*$^{-/-}$ LSKs analyzed for surface expression of Flt3 and CCR9. CCR9 expression in *Tcfe2a*$^{+/+}$ LK^{-}S cells is shown for comparison. One of three experiments is shown.

(E) Absolute number of CCR9^{+} LMPPs in the BM (femurs and tibias) of *Tcfe2a*$^{+/+}$, *Tcfe2a*$^{+/-}$, and *Tcfe2a*$^{-/-}$ mice. Error bars represent the mean ± SD; * $p < 0.05$.

expressed in *Tcfe2a*$^{+/+}$ and *Tcfe2a*$^{-/-}$ LMPPs, and there was no compensatory increase in the number of Flt3^{-} or Flt3^{lo} LSKs in *Tcfe2a*$^{-/-}$ mice, as might be predicted if LMPPs lacking Flt3 were present. Moreover, ectopic expression of E2A in *Tcfe2a*$^{-/-}$ progenitors does not alter *Flt3* mRNA, even though *Ccr9* mRNA is induced. Using loss of VCAM-1 as a distinguishing feature of LMPPs, we also found that LMPPs were reduced in *Tcfe2a*$^{-/-}$ mice (Lai and Kondo, 2007; Lai et al., 2005). Therefore, the loss of Flt3^{hi} LSKs in *Tcfe2a*$^{+/-}$ and *Tcfe2a*$^{-/-}$ mice reflects a decline in LMPP numbers. Although E2A proteins do not regulate *Flt3* mRNA, it is possible that Flt3 signaling regulates E2A function. Flt3 could regulate E2A by phosphorylation or indirectly through Flt3-dependent downregulation of class II bHLH proteins, such as stem cell leukemia (SCL) protein, also known as T lymphocyte acute lymphoblastic leukemia protein 1 (TAL1) (Adolfsson et al., 2005; Neufeld et al., 2000).

E2A proteins clearly play a major role in the development of LMPPs. However, a small number of LMPPs develop in *Tcfe2a*$^{+/-}$ and *Tcfe2a*$^{-/-}$ mice, and these cells are able to generate T lymphocytes, but not B lymphocytes, in vivo. A possible explanation for the development of these few LMPPs is that they rely on E protein activity provided by the E proteins HEB and E2-2. The E proteins are functionally redundant and can cooperate with other transcription factors to activate E2A target genes, even when expressed at low concentrations (Seet et al., 2004; Zhuang et al., 1998). Nonetheless, a number of lymphoid genes fail to be appropriately expressed in *Tcfe2a*$^{-/-}$ LMPPs, including *Rag1*, *Il7r*, *Dntt*, *Ets1*, and *Ccr9*. It is unlikely that these particular genes regulate the development of LMPPs because LMPPs develop in mice that lack *Rag1*, *Ccr9*, *Il7r*, or *Ets1* (B.L.K., unpublished data and Schwarz et al. [2007]). Regardless, our findings indicate that within LMPPs, E2A is required for proper expression of a large subset of lymphoid genes. Interestingly, a subset of these genes can be detected in HSCs and are E2A dependent, indicating that the lymphoid gene program is beginning priming at a substantially earlier developmental stage than previously appreciated. This observation is consistent with other studies that have identified lineage-associated transcripts in HSCs (Manaia et al., 2000; Ye et al., 2003; Yoshida et al., 2008) and raises the possibility that this subset of E2A-dependent genes may play a role in specifying the lymphoid fate (Georgopoulos, 2002).

In addition to regulating lymphoid-associated genes, E2A was necessary to prevent expression of a set of genes not normally found in LMPPs. Interestingly, a subset of these genes was also increased in *E2A*$^{-/-}$ HSCs. This observation, in conjunction with the decline in MPP and LMPP numbers, indicates that the function of E2A extends beyond lymphoid priming. Indeed, loss of E2A was associated with an increased proliferation of HSCs, MPPs, and LMPPs, as well as increased clongenic potential and GMP differentiation in LMPPs, effects that are unlikely to be attributed to a lack of lymphoid priming. The loss of LMPPs in *Tcfe2a*$^{+/-}$ and *Tcfe2a*$^{-/-}$ mice did not appear to be due to a failure of expansion or increased cell death. Rather, fewer cells were undergoing lymphoid specification and instead adopted the GMP fate. A role for E2A proteins in restricting GMP

Table 1. E2A-Dependent Lymphoid Gene Expression in HSCs

Probe Set	Gene Name	$E2A^{+/+}$ HSC (mean)*	$E2A^{-/-}$ HSC (mean)*	Fold Decrease in $E2A^{-/-}$ HSC (95% confidence interval)
1449757_x_at	Dntt	1370.74	135.61	10.11 (8.92–11.66)
1450545_a_at	Dntt	302.53	37.24	8.12 (6.43–10.80)
1427351_s_at	Igh-6	662.31	112.67	5.88 (5.37–6.46)
1427329_a_at	Igh-6	262.57	50.12	5.24 (4.92–5.6)
1422851_at	Hmga2	291.41	79.50	3.67 (2.99–4.74)
1420805_at	Mylc2pl	87.59	25.35	3.45 (3.26–3.69)
1455570_x_at	Cnn3	132.12	40.25	3.28 (2.87–3.81)
1436759_x_at	Cnn3	139.58	42.86	3.26 (2.90–3.68)
1449310_at	Ptger2	51.64	16.57	3.12 (2.47–3.86)
1436836_x_at	Cnn3	186.31	62.16	3.00 (2.62–3.43)
1450780_s_at	Hmga2	218.78	73.10	2.99 (2.49–3.73)
1426725_s_at	Ets1	143.95	72.09	2.00 (1.62–2.46)
1419872_at	Csf1r	229.91	116.53	1.97 (1.73–2.22)
1418634_at	Notch1	608.16	311.76	1.95 (1.82–2.10)
Probe Set	Gene Name	$E2A^{+/+}$ HSC (mean)*	$E2A^{-/-}$ HSC (mean)*	Fold increase in $E2A^{-/-}$ HSC (95% confidence interval)
1434465_x_at	Vldlr	369.8	757.21	2.05 (1.66–2.44)
1439814_at	Atp8b4	199.22	415.49	2.09 (1.82–2.44)
1435893_at	Vldlr	127.66	275.66	2.16 (1.66–3.08)
1434278_at	Mtm1	229.19	505.02	2.21 (1.96–2.52)
1417900_a_at	Vldlr	92.11	210.58	2.29 (1.95–2.75)
1457139_at	Auts2	41.51	133.81	3.22 (2.71–3.94)

A total of 30 unique genes were differentially expressed in $Tcfe2a^{-/-}$ HSCs ($Flt3^-$ LSKs) as compared to $Tcfe2a^{+/+}$ HSCs (21 decreased and 9 increased).
* Mean of two replicate samples.

differentiation is consistent with a previous report demonstrating that E12 can induce lymphoid gene expression and repress myeloid traits in a macrophage cell line (Kee and Murre, 1998) and our recent finding that ectopic expression of E12 in $Tcfe2a^{-/-}$ MPPs promotes B lymphocyte development and represses macrophage development in vitro (Bhalla et al., 2008). Therefore, E2A proteins, possibly in collaboration with other lymphoid transcription factors, promote lymphoid specification.

Although E2A proteins promote lymphoid specification, they are not required for restriction of Mk potential in LMPPs. Therefore, E2A proteins do not promote lymphomyeloid specification at the expense of erythromyeloid specficiation, even though CMPs and MEPs are slightly increased in $Tcfe2a^{-/-}$ mice. This increase may be a result of the increased proliferation of $Tcfe2a^{-/-}$ HSCs, which could lead to greater erythromyeloid output over time. Interestingly, however, E2A proteins are highly

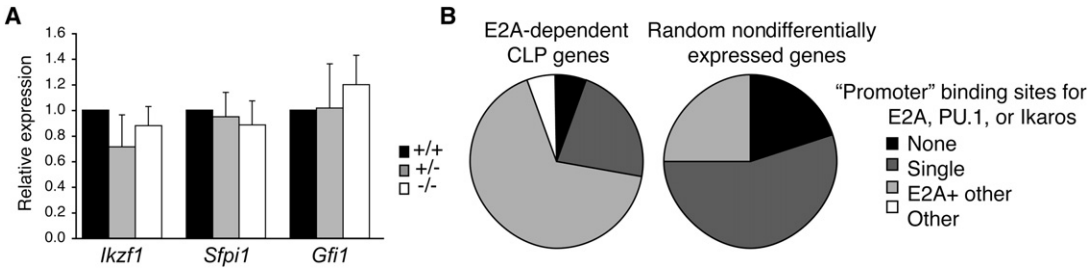

Figure 5. Expression of Key Lymphoid Transcription Factors in the Absence of E2A
(A) QPCR for *Ikzf1*, *Sfpi1*, and *Gfi1* mRNA in purified $Tcfe2a^{+/+}$, $Tcfe2a^{+/-}$, and $Tcfe2a^{-/-}$ LMPPs (normalized to *Hprt* and amount in $Tcfe2a^{+/+}$ LMPPs set to 1). Error bars represent the mean ± SD.
(B) Distribution of conserved potential E2A, Ikaros, and PU.1 binding sites in the promoters (−7.5 kb to +2.5 kb) of E2A-dependent lymphoid-associated genes or a set of randomly selected LMPP genes whose expression is not E2A dependent. Binding sites were identified with rVISTA through the ECR Browser. "None" indicates no binding sites for E2A, PU.1, or Ikaros (black). "Single" indicates a binding site for only one factor; in the E2A-dependent case, these were all E2A-binding sites, whereas in the random genes, they were equally mixed (dark gray). "E2A+other" indicates a binding site for E2A with PU.1 and/or Ikaros (light gray). "Other" indicates a binding site for PU.1 and/or Ikaros but not E2A (white).

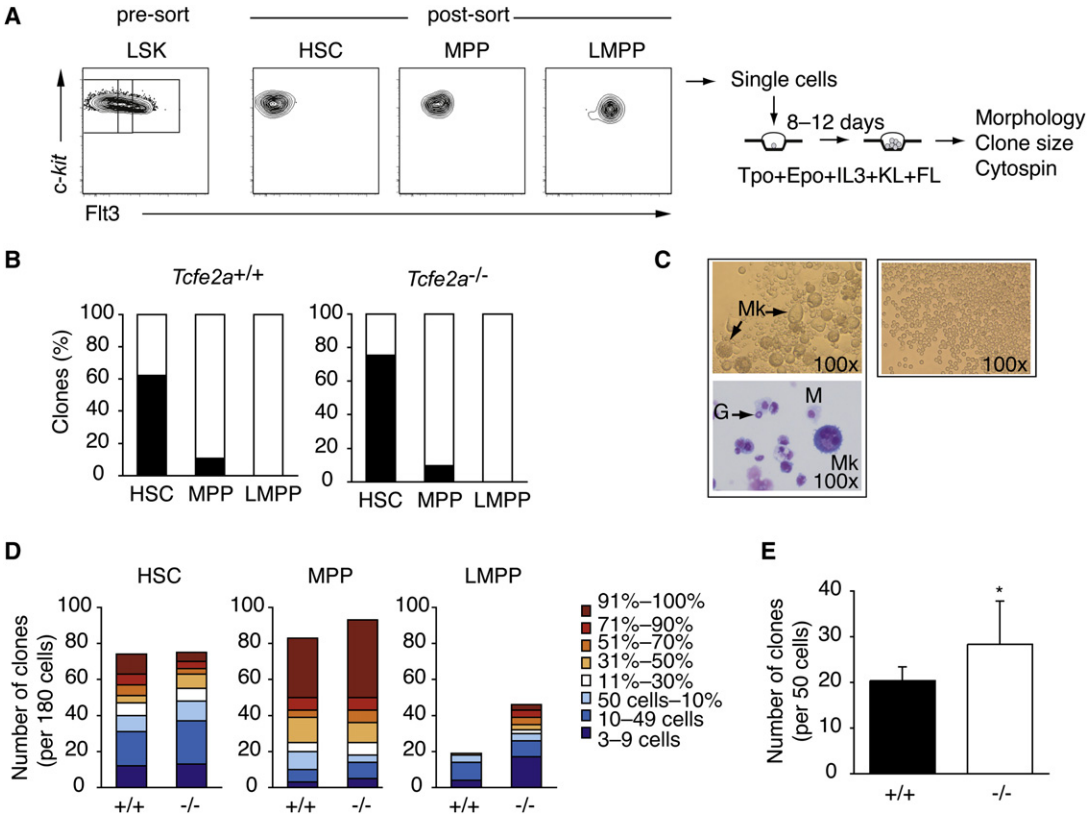

Figure 6. *Tcfe2a*$^{-/-}$ LMPPs Restrict the MEP Fate but Show Increased Myeloid Clonogenic Potential

(A) HSCs, MPPs, and LMPPs were isolated from the BM LSK population by cell sorting, and single cells were seeded in wells of Terasaki plates in conditions supporting multilineage myeloid differentiation.

(B) Frequency of *Tcfe2a*$^{+/+}$ and *Tcfe2a*$^{-/-}$ clones giving Mk containing (black) or GM only (white) colonies in vitro. One of four representative experiments is shown.

(C) Representative examples of colonies that were positive (left) or negative (right) for the presence of megakaryocytes (Mk). Wright-Giemsa staining of the Mk-containing clone (bottom). "G" denotes granulocyte; "M" denotes macrophage.

(D) Number of clones generated from 180 single-cell cultures of *Tcfe2a*$^{+/+}$ or *Tcfe2a*$^{-/-}$ HSCs, MPPs, or LMPPs. Color-coding from blue to red represents the size of colonies from smallest (three to nine cells) to largest (90%–100% of well). The majority of MPP- and all LMPP-derived colonies were GM (see Figure S4A). One of three representative experiments is shown.

(E) Number of clonable *Tcfe2a*$^{+/+}$ (black) and *Tcfe2a*$^{-/-}$ (white) LSKFlt3^{+} progenitors determined at day 7 of culture in methylcellulose (mean of three replicate wells ± SD; *$p < 0.05$). One of three representative experiments is shown.

expressed in erythromyeloid progenitors and can function as dimerizing partners for SCL and Lyl1, which play an essential role in Mk and E differentiation (Lecuyer and Hoang, 2004). Therefore, appropriate regulation of E2A-protein homodimer and heterodimer formation may play a major role in allowing the differentiation of these two pathways downstream of HSCs. At the present time, it is not known whether there is a single progenitor cell that makes a binary decision regarding the erythromyeloid or lymphomyeloid fate, and recent studies suggest that these decisions may be temporally segregated (Arinobu et al., 2007). Therefore, considerable experimentation is still required to reveal the basic mechanisms dissociating these two branches of the hematopoietic system and the role of lymphoid-promoting transcription factors in restricting MEP differentiation.

Our data also provide insight into the relationship between E2A and Notch1 and the regulation of T lymphocyte specification. We showed that E2A was required in vivo for optimal expression of *Notch1* mRNA in LMPPs and HSCs. Although the reduced amount of *Notch1* does not appear to prevent T cell development, it may nonetheless decrease the "fitness" of these ETPs in vivo, as observed with *Notch1*$^{+/-}$ ETPs (Tan et al., 2005). We found that the number of ETPs in *Tcfe2a*$^{+/-}$ and *Tcfe2a*$^{-/-}$ mice was reduced in proportion to the decrease in LMPPs, given the potential amplifying effect of the lack of CCR9 (Schwarz et al., 2007). Therefore, our data suggest that the primary cause of the reduced number of DN thymocytes in *Tcfe2a*$^{+/-}$ and *Tcfe2a*$^{-/-}$ mice is the failure to produce sufficient BM-derived lymphoid-primed progenitors. However, the fact that *Tcfe2a*$^{-/-}$ thymocytes frequently fail to recover to WT numbers by the DN3 stages suggests that there may be additional defects in these cells that prevent appropriate T cell development.

The data we have presented indicate that E2A proteins function in the transcriptional network that specifies the lymphomyeloid fate in multipotent progenitors and in the lymphoid-priming within these cells and their precursors. These newly identified functions for E2A, together with its role in regulation of B and T

lymphocyte-specific gene expression, resembles the known functions of the *Drosophila* homolog of E2A, *daughterless*, in neurogenesis, during which it is required to establish the identity of cells with potential to develop into neuronal lineages (the proneural field) and subsequently functions within the proneural field to activate lineage-specifying genes (Bertrand et al., 2002). Similar functions have been described for class II bHLH proteins, which require E proteins to mediate transcription, in determination and differentiation of olfactory neurons in mammals (Cau et al., 2002). Therefore, the E proteins may play universal functions in cell-fate determination and differentiation.

EXPERIMENTAL PROCEDURES

Mice

Mice were housed at the University of Chicago Animal Resource Center, and experiments were performed in accordance with the guidelines of the National Institutes of Health and protocols approved by the Institutional Animal Care and Use Committee. C57Bl/6 *E47*$^{-/-}$ mice (here referred to as *Tcfe2a*$^{-/-}$) and genotyping protocols have been described previously (Bain et al., 1997b). Mice were analyzed between 6 and 12 weeks of age.

Flow Cytometry and Sorting

Cells were harvested from thymus or BM and stained with specific combinations of antibodies (see Table S1) purchased from BD Biosciences and eBiosciences, with the exception of CCR9 (R&D Systems). Lineage antibodies were biotinylated (revealed with streptavidin-PECy5.5), and the remainders were conjugated to FITC, PE, PE-Cy7, or APC. Cells were analyzed with a FACS Canto and FlowJo (Tree Star) software or sorted with a FACS Aria. For sorting, lineage^{+} cells were first depleted by magnetic-activated cell separation with streptavidin MicroBeads (Miltenyi Biotec) according to the manufacturer's instructions. Dead cells were stained with PI and excluded by electronic gating. Intracellular staining was performed as described previously with purified hE47 monoclonal antibody (BD Biosciences) (Engel et al., 2001). We used negative-staining controls to distinguish negative and positive populations. LSK gates were determined with the strategy described by Kondo et al. (1997). Flt3 gates were determined with the strategy described by Adolfsson et al. (2005).

Determination of Cell Numbers in Each Progenitor Population

We determined the absolute number of viable cells in the BM (two femurs and two tibias) or thymus by trypan blue exclusion. We multiplied this number by the percentage of Lin^{-} cells in the viable cell gate (PI^{-}) and then by the percentage of cells in each subgate thereafter to determine the absolute number of progenitors in each subpopulation per mouse. For BM progenitors, we then further normalized this number to 50 × 10^{6} total BM cells to minimize differences due to animal size. Relative cell numbers were calculated by dividing the number of cells in each subpopulation by the number of cells in that same subpopulation in WT mice (therefore, WT = 1 for each cell population and in each set of mice analyzed). Statistical significance was assessed by the Student's t test.

In Vivo BrdU-Incorporation Assay

Mice were injected intraperitoneally with 1 mg BrdU per 6 g of body weight (BD Biosciences) 24 hr and 12 hr prior to analysis of BM subpopulations by flow cytometry. BrdU staining was performed with the FITC BrdU Flow Kit (BD Biosciences) according to the manufacturer's instructions.

In Vitro Apoptosis Assay

Sorted HSC, MPP, and LMPP were cultured in media lacking serum for 20 hr. Staining was performed with the Annexin-V-FITC Apoptosis Detection Kit (BD Biosciences) according to the manufacturer's instructions.

Affymetrix Gene Expression and Data Analysis

RNA was extracted with the RNeasy Micro Kit (QIAGEN), labeled, amplified, and hybridized to MOE430 2.0 Affymetrix gene-expression arrays according to Affymetrix standard protocols. Probe-level expression values were calculated with RMA (Irizarry et al., 2003). Further analysis was performed with dChip (www.dchip.org).

Transcription-factor binding sites conserved between human and mouse genomic sequences were identified in the promoters of E2A-dependent lymphoid-associated genes and a set of randomly selected genes with rVISTA (Dubchak and Ryaboy, 2006). The promoters were defined as the region −7.5 kb to +2.5 kb of the transcription start site as defined by gene annotation in the ECRBrowser (http://ecrbrowser.dcode.org/). The E2A-dependent lymphoid-associated genes examined were as follows: *Apcdd1*, *Arpp21*, *Blnk*, *Ccr9*, *Cnn3*, *Dntt*, *Ets1*, *Fgf13*, *Gsn*, *Hmga2*, *Il7r*, *Klf3*, *Mylc2pl*, *Notch1*, *Ptger2*, *Rag1*, *Slc9a9*, and *Slc27a2*. The randomly selected genes were as follows: *Appbp1*, *Cdk4*, *Cdk9*, *Cog2*, *Ctsc*, *Entpd5*, *Gdi2*, *Hnrpa2*, *Mrps14*, *Ndufb7*, *Rplp1*, *Rpl14*, *sdfr1*, *Smc6l1*, *Snrpa1*, *Snsx3*, *Timeless*, *Tpd52l2*, *Uba52*, and *Ywhab*.

Quantitative PCR

RNA was extracted with the RNeasy Micro Kit and cDNA was prepared with random primers and SuperScriptIII reverse transcriptase (Invitrogen), according to the manufacturer's instructions. QPCR reactions were performed in triplicate with iQ SYBR Green Supermix (Biorad) and detected by MyiQ Single Color Real-Time PCR System (Biorad). Most primers (see Table S2) amplify multi-exon sequences; for the remainder, DNaseI (QIAGEN) treatment and a nonRT negative control were included.

Retroviral Transduction

Sorted Lin^{-}*c-kit*^{high}CD27^{+} fetal-liver progenitors were spinoculated (2 hr at 2500 rpm) in the presence of 5 μg/ml polybrene with pCS and pCS-E47-ER (Sayegh et al., 2003) retroviral supernatants produced in Plat-E cells. Transduced cells were cultured on OP9-DL1 stroma in the presence of *c-kit*-ligand, Flt3-ligand, IL7, and 1 μM 4-hydroxytamoxifen for 36 hr.

In Vitro Evaluation of Erythromyeloid Differentiation Potential

Single BM HSCs, MPPs, and LMPPs were seeded in Terasaki plates (180 cells per population in each experiment) and cultured as previously described (Mansson et al., 2007). Wells were scored with an inverted light microscope on days 9–12 of culture for clonal growth, clone size, and frequency of Mk. Mk detection was confirmed by Wright-Giemsa staining of cytospin preparations. Fifty BM Flt3^{+} LSK cells were seeded per well in six-well plates, in 1.1 ml MethoCult 03434 (StemCell Technologies) supplemented with 50 ng/mL Flt3-ligand and 10 ng/mL thrombopoietin (PeproTech). Clonal growth was evaluated on day 7.

ACCESSION NUMBERS

Array data are accessible through the gene expression omnibus (GEO; GSE8407, GSE7302, and GSE11982).

SUPPLEMENTAL DATA

Supplemental Data include four figures and two tables and can be found with this article online at http://www.immunity.com/cgi/content/full/29/2/217/DC1/.

ACKNOWLEDGMENTS

We thank the members of the Flow Cytometry Core Facility at the University of Chicago for cell sorting and the Microarray facility at Lund University and Katia Georgopoulos for *Ikaros* primer sequences. We are grateful to members of the Kee Lab for helpful discussions. S.D. was supported by the University of Chicago Committee on Cancer Biology. This work was supported by the National Institutes of Health and the National Cancer Institute (CA99978).

Received: November 26, 2007
Revised: May 12, 2008
Accepted: May 30, 2008
Published online: July 31, 2008

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